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Frequency and distance of transposition of a modified *Dissociation* element in transgenic tobacco

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Effective transposon tagging with the *Ac/Ds* system in heterologous plant species relies on the accomplishment of a potentially high transposon-induced mutation frequency. The primary parameters that determine the mutation frequency include the transposition frequency and the transposition distance. In addition, the development of a generally applicable transposon tagging strategy requires predictable transposition behaviour. We systematically analysed *Ds* transposition frequencies and *Ds* transposition distances in tobacco. An artificial *Ds* element was engineered with reporter genes that allowed transposon excision and integration to be monitored visually. To analyse the variability of *Ds* transposition between different tobacco lines, eight single copy T-DNA transformants were selected. For *trans*-activation of the *Ds* elements, different *Ac* lines were used carrying an unmodified Ac^+ element, an immobilized *sAc* element and a stable *Ac* element under the control of a heterologous chalcone synthase (*chsA*) promoter. With all *Ac* elements, each *Ds* line showed characteristic and heritable variegation patterns at the seedling level. Similar *Ds* line-specificity was observed for the frequency by which *Ds* transpositions were germinally transmitted, as well as for the distances of the *Ds* transpositions. The *sAc* element induced transposition of *Ds* late in plant development, resulting in low germinal transposition frequencies (0.37%) and high incidences of independent transposition (83%). The majority of these *Ds* elements (58%) transposed to genetically closed linked sites (≤10 cM).

Keywords: heterologous transposon tagging; *Ac/Ds* system; visual transposition assays; position effects; *Nicotiana tabacum*

Introduction

The *Activator* (*Ac*)/*Dissociation* (*Ds*) transposon system from maize (McClintock, 1951) provides a powerful means for gene tagging in heterologous plant species. The autonomous *Ac* element consists of a transposase gene and discrete termini that are required for transposition to occur (Kunze *et al.*, 1987; Coupland *et al.*, 1988, 1989). Most *Ds* elements are *Ac*-derivatives with internal deletions in the transposase gene and, consequently, are non-autonomous. The *Ac/Ds* transposition behaviour in heterologous plant species has been shown to represent that in maize (Hehl and Baker, 1990; Jones *et al.*, 1990). Transposition takes place non-replicatively, is dependent of the transposase gene dosage, occurs predominantly to genetically linked sites and upon integration an 8 bp duplication of the target site is created. One advantage of transposon tagging over other approaches to isolate genes with unknown products, such as T-DNA tagging and map-based cloning, is that no genetic complementation analysis is required: the authenticity of the insertion mutant can be verified by reversion analysis (Walbot, 1992). Unstable phenotypes caused by germinal or somatic reversion to wild-type present evidence that the gene is mutated by a transposon. Part of the gene flanking the transposon can readily be cloned by inverse PCR (Earp *et al.*, 1990) or plasmid rescue (Rommens *et al.*, 1992a) and subsequently, can directly be used for the isolation of the corresponding complementary DNA.

Heterologous *Ac/Ds* transposon tagging strategies have been developed in a variety of plant species (Haring *et*

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al., 1991; Van der Biezen *et al.*, 1994). The *Ac* components, functioning as transposase donors, were stabilized by deleting part of the *cis*-required sequences. The regulatory sequences of the transposase genes were often modified to increase the effectiveness of *Ds trans*-activation. The constructs with the *Ds* components were equipped with selectable or detectable markers to monitor transposition in plants. By non-targeted transposon tagging, genes were tagged that corresponded to any phenotype that was correlated with a transposon insertion (Bancroft *et al.*, 1993; Long *et al.*, 1993; Chuck *et al.*, 1993; Van der Biezen *et al.*, 1996). The effectiveness of these experiments depended on the selection efficiency of plants that carried transposition events. By targeted transposon tagging, the plant crosses were designed to isolate one specific gene-of-interest (Jones *et al.*, 1994; Whitham *et al.*, 1994; James *et al.*, 1995; Lawrence *et al.*, 1995). The efficiency of this procedure depended on the transposon activity and the genetic distance of the transposons to the target genes. Close linkage between the original transposon location and the target gene increased the mutation frequency considerably.

With respect to the mutagenic potential of the *Ac*/*Ds* system, we recently used a mathematical approach to dissect the transposition behaviour into its components (Van der Biezen *et al.*, 1994). To predict statistically the transposon-induced mutation frequency in heterologous hosts, the contribution of the involved parameters was estimated. In this paper, we systematically investigated the two major parameters that determine the efficiency of transposon tagging, including the frequency of somatic and germinal *Ds* transposition and the *Ds* transposition distance. Also, the variation of these transposition parameters between different tobacco lines was analysed. To these ends, a T-DNA construct with an artificial *Ds* element was designed comprising plasmid rescue sequences and reporter genes that allowed excision and reinsertion to be visually monitored. Eight transgenic tobacco lines were selected harbouring single copy T-DNA insertions. Following *trans*-activation of *Ds* by three different *Ac* constructs, plants with unique, germinally transmitted stable transposed *Ds* elements were identified and the transposition distances determined by segregation analysis of the reporter genes. The *Ds* lines showed characteristic transposition behaviour for the timing and frequency of *Ds* excision during embryogenesis, for the germinal *Ds* transposition frequencies, and for the distance of *Ds* transpositions. Furthermore, seedling assays showed that *Ds* was much more active during embryogenesis than at mature stages. In mature plants the *Ds* transpositions took place late in development, resulting in low germinal transposition frequencies and high incidences of independent transposition events. The *Ds* elements had a strong preference for transposition to genetically closely linked sites.

Materials and methods

Recombinant cloning of Albatross

To construct an artificial *Ds* element with the short 5′ and 3′ termini of *Ac* (Behrens *et al.*, 1984), PCR amplification was employed using the *act3a*/*act3b* primers (1–194) and the *act5a*/*act5b* primers (4234–4565). *Ac* elements contain imperfect terminal repeats (TIRs, $^{T}/_{c}$AGGGATGAAA), while *Ds* elements have perfect TIRs (both terminal bases are Ts). To maintain the homology with natural *Ds* elements, the *act5a* primer was designed such that it changed the terminal C into a T. Both PCR fragments were cloned in pSU20 (Bartolomé *et al.*, 1991) with the TIRs directed towards each other resulting in plasmid pEB10. The DNA sequence of the 0.5 kb *Ds* insert of pEB10 was verified by double-strand dideoxy automatic sequencing (Applied Biosystems Model 373A). The pSU20 vector was chosen for potential application of plasmid rescue of *Ds*-flanking plant DNA because of the small size (2.3 kb), the compatibility with other plasmids for persistence in bacterial hosts (p15A origin of replication), and the presence of the bacterial chloramphenicol resistance gene (*CAT*). The pEB10 plasmid was linearized by cutting between the TIRs and ligated between the *CaMV-35S* promoter and the *TP:SPEC* gene from pSLJ2524 (Scofield *et al.*, 1994). Subsequently, the total fragment was inserted between the T-DNA borders of pMOG22 (Mogen, Leiden, Netherlands), from which the *35S:HPT* gene was deleted first. Finally, the *NPTII* gene (McBride and Summerfelt, 1990) was inserted between pSU20 and the 5′ *Ds* terminus. The total T-DNA construct was named *Albatross* (Fig. 1) and contained a *Ds* element of 5.3 kb that is of comparable size to *Activator* (*Ac*) elements (4.6 kb). Recombinant clones and binary vectors were constructed by standard procedures (Sambrook *et al.*, 1989) in *Escherichia coli* strain JM101 and transferred to *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983). Details of the cloning procedures are available on request.

Plant materials, plant transformations and plant crosses

SLJ10512B tobacco plants carried a single T-DNA insertion with a stabilized *Activator* element (*sAc*) of which the 177 terminal bp at the 3′ end had been deleted, a *GUS* reporter gene and a *NPTII* gene to select transformants (Scofield *et al.*, 1992). Plants with the *Ac* transposase construct driven by the flower-specific chalcone synthase A (*chsA*) promoter from *Petunia hybrida* (*chs:Ac*) were described by Rommens *et al.* (1992b), and the plants with the wild-type (unmodified) and transpositionally active *Ac* element (Ac^{+}) were described by Haring *et al.* (1989).

Tobacco Petit Havana SR_1 leaf discs were transformed with the T-DNAs of *Albatross* and pSLJ4731 (Jones *et al.*, 1992) by *Agrobacterium*-mediated transformation

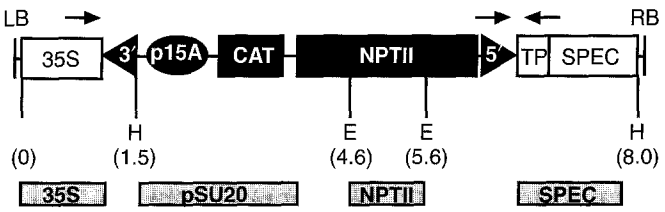

Fig. 1. Map of the *Albatross* T-DNA comprising an artificial *Ds* element of 5.3 kb (shown in black). The *Ds* element contained (1) the short 5′ (331 bp) and 3′ (194 bp) termini of *Ac*, (2) a bacterial chloramphenicol resistance gene (*CAT*) and a bacterial origin of replication (p15A) permitting plasmid rescue of flanking plant DNA, and (3) a neomycin phosphotransferase gene (*NPTII*) for kanamycin resistance that functioned as plant transformation marker and *Ds* insertion marker. *NPTII* was regulated by the promoter and the terminator of the mannopine synthase gene (*mas5′:NPTII:mas3′*). To monitor transposon excision, *Ds* was cloned between the cauliflower mosaic virus *CaMV* 35S promoter and the spectinomycin resistance gene (*SPEC*). For cell-autonomous action, the *SPEC* gene was preceded by a chloroplast transit peptide sequence (*TP*) from *P. hybrida* and concluded by the nopaline synthase termination sequence (*35S:TP:SPEC:nos3′*). For transfer to the plant genome the transposon-construct was flanked by the left-(LB) and right borders (RB) of the T-DNA of *Agrobacterium*. The positions (kb) of the *Eco*RI (E) and *Hin*dIII (H) restriction enzyme recognition sites are shown. The annealing sites of the oligonucleotides used as primers in polymerase chain reactions (PCR) are presented as arrows above the construct. Hybridization probes used in Southern analyses are shown as boxes below the construct.

(Horsch *et al.*, 1985). Kanamycin-resistant (100 mg l^{-1}) *Albatross* transformants were designated *Alb* followed by a number for each independent transformant and a letter to distinguish siblings.

All crosses were performed in the greenhouse using standard emasculation and pollination techniques. Only the results of crosses with plants that were homozygous for the respective T-DNAs are reported. The genotypes were determined by testing self progenies for segregation of kanamycin (Km) resistance in seedling assays. The F_1s were made by crossing the *Ac* parents (*sAc*, *chs:Ac*, *Ac*$^+$) with the *Albatross* lines as the pistillate parents. The F_1 plants were hemizygous for *Ac* and *Ds* containing T-DNAs and were outcrossed to produce OC_1 progenies. Reciprocal outcrosses were made; however, generally the non-transgenic Petit Havana SR_1 cultivar was used as the staminate parent. To analyse the inheritance of (independent) transposition events in the OC_1, the position of each outcross on the specific branch, the specific inflorescence and the specific flower was recorded (Keller *et al.*, 1993). Finally, the selected OC_1 individuals were self-pollinated to produce OC_1S_1 families. Plants were grown in pots under standard Dutch greenhouse conditions (20°C, 60% humidity, 3 klx).

Phenotypic seedling assays for monitoring transposition

For aseptic assays, seeds were dipped in 70% ethanol for 30 s, soaked in 1% NaClO (w/v) for 20 min and rinsed in sterile water. Following this sterilisation procedure, 100–600 seedlings were plated in Petri dishes containing Murashige and Skoog (MS) medium pH 5.7 with 1.5% sucrose, 0.8% agar (Difco), spectinomycin (100 mg l^{-1}) and/or kanamycin (200 mg l^{-1}) (Duchefa) and placed in a climate cabin under standard conditions (16 h, 3 klx, 22°C). After 2–3 weeks the antibiotic resistant and sensitive seedlings were counted, and the selected seedlings subjected to GUS analysis and grown in soil to mature plants for genetic and molecular analysis.

Histochemical GUS analyses were done by placing the roots of seedlings in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) in 50 mM $NaHPO_4$ pH 7. The seedlings were maintained at 100% humidity to prevent dehydration and placed at 25°C for 2–4 h. Seedlings containing the *GUS* gene showed blue coloration of the roots. Because seedlings showing no GUS activity were selected for further analysis, the absence of the *sAc* T-DNA (SLJ10512) was verified in leaf tissue extracts from greenhouse grown plants. First, fluorometric GUS analyses with 1 mM 4-methyl-umbelliferyl-β-D-glucuronide (MUG) were done (Jefferson *et al.*, 1987) and, secondly genomic DNA was subjected to PCR with *Ac*-specific primers (*ac6*/*ac7*).

Tobacco seedlings that were germinated on medium containing spectinomycin (Sp) developed bleached chloroplasts and, as a consequence, had white (W) cotyledons. However, when transformed with the *SPEC* gene (*aadA*), seedlings appeared fully green (FG) on this medium. For cell-autonomous action, *SPEC* was preceded by a chloroplast transit peptide sequence (*TP*) from *P. hybrida* (Scofield *et al.*, 1994). The *Ds* element cloned between the promoter and *TP:SPEC* prevented transcription of this gene but expression was restored following excision of *Ds*. Somatic excisions resulted in variegation (V) patterns of Sp-resistant green spots on the Sp-sensitive white background of the cotyledons. The somatic excision patterns were classified according to the degree of green variegation: fully green seedlings were totally green (FG); highly variegated (HV) seedlings were almost totally green with some small white flecks on the surface lamina or in inner cell layers; medium variegated (MV) seedlings had 3–10 distinct green spots; and lowly variegated (LV) seedlings had 1–2 small green spots. Germinally transmitted *Ds* excisions resulted in seedlings carrying the event in all cells and hence were fully green (FG) when germinated in the presence of Sp.

In seedling assays, kanamycin (Km) resistance was indicative of the presence of the *Ds* element (*NPTII*) and Sp resistance was indicative of the presence of the

Albatross T-DNA (*SPEC*) from which a *Ds* element had been excised (empty donor site, EDS). In medium with Sp and Km, the presence of both markers resulted in fully green (FG) seedlings, the absence of both markers resulted in white (W) seedlings. Seedlings with only an EDS T-DNA and no *Ds* (Sp^R/Km^S) developed yellow (Y) cotyledons on Sp/Km medium. As control for the transposition reporter genes and the phenotypic seedling assays, various tobacco lines were used that carried none, either one, or both markers. Self seedlings of these transformants were germinated in the presence of both Sp and Km. Seedlings with the pSLJ4731 construct (*SPEC* and *NPTII*; Jones *et al.*, 1992) were fully green (FG) (Sp^R/Km^R); pEB34 plants (pMOG22 with *SPEC* from pSLJ4731) were yellow (Y) (Sp^R/Km^S); *Albatross* and SLJ10512 lines were white (W) (Sp^S/Km^R); and seedlings of the non-transgenic cultivar Petit Havana SR_1 were also white (W) (Sp^S/Km^S).

Calculation of the transposition frequencies and the transposition distance

Somatic excision frequencies in F_1 progenies (all hemizygous for both and *Ac* and *Ds* T-DNAs) were calculated by dividing the number of seedlings showing Sp resistance (FG or V) by the total number of seedlings. The number of FG seedlings in OC_1 progenies was a measure for the germinal excision frequency and was based on the proportion that inherited the *Ds* T-DNA, i.e. half of the total population. Germinal transposition events (i.e. excision followed by reinsertion) resulted in FG OC_1 seedlings when germinated on double-selective Sp/Km medium. However, FG seedlings (Sp^R/Km^R) were not necessarily the consequence of germinal transposition, because Km resistance could also be conferred by the *sAc* T-DNA (SLJ10512) which segregated in OC_1 progenies and, moreover, Sp resistance could also be the result of somatic excision. To select seedlings indisputably harbouring a germinally excised and reinserted *Ds* element, FG seedlings were selected that did not contain the SLJ10512 T-DNA (*sAc*::*NPTII*::*GUS*) by analysis of GUS activity. In this way, somatic excision was excluded and Km resistance could only be conferred by the *NPTII* gene within the *Ds* element. This approach had the concomitant advantage that stable insertions were selected which allowed reliable determination of the transposition distances in self progenies (OC_1S_1). To avoid underestimation of the germinal transposition frequencies, selection against *sAc* required that this SLJ10512 T-DNA was unlinked (in repulsion) to the *Albatross* T-DNA. Segregation analysis of OC_1 progenies by Km and Sp/Km assays indeed indicated that all eight *Albatross* T-DNAs were unlinked to the *sAc* T-DNA (SLJ10512). For the calculation of the germinal transposition frequencies only half of the FG seedlings could unambiguously be recognized (1/2 without *sAc*), and therefore, the proportion of FG/GUS^- seedlings was based on half of the number of seedlings that potentially could inherit a *Ds* element (i.e. half of the total seedlings), thus 1/4 of the total OC_1 population. This OC_1 population size was based on the number of variegated (V) seedlings which was 1/4 of the total number of OC_1 seedlings (see Results). Consequently, the germinal transposition frequency was calculated by (No of FG & GUS^-)/(No of V). In OC_1 progenies of *Alb*15, the sizes of the OC_1 populations were determined by counting all seedlings.

The distance of transposition was expressed as the recombination frequency between the *Ds* element and the original donor site, the *Albatross* T-DNA, converted to centiMorgans (cM). Recombination between the *Ds* element and the T-DNA resulted in separation of the respective *NPTII* (Km^R) and *SPEC* (Sp^R) markers which were scored in OC_1S_1 progenies. However, only half of the recombinants could be recognised on Sp/Km medium: those that contained the T-DNA and no *Ds* element (Sp^R/Km^S) and, consequently, developed yellow (Y) cotyledons. The other class of recombinants only carried the *Ds* element and no T-DNA (Sp^S/Km^R), and hence developed white (W) cotyledons which could not be distinguished from non-transgenic white (W) seedlings (Sp^S/Km^S). Therefore, the proportion of recombinants (Y) was based on half of the total OC_1S_1 population. When significant linkage was oberved ($P < 0.01$ if $\chi^2 > 11.32$), the recombinant fractions (r) were calculated by (No of Y)/($\frac{1}{2}$ × total No). Subsequently, the genetic distances were converted to cM using Haldane's mapping function: $(-\ln(1 - 2r)/2) \times 100$.

DNA isolation and Southern analysis

DNA isolations and Southern hybridizations were done as described before (Van der Biezen *et al.*, 1995). Five radiolabelled probes were used: SPEC (*Xho*I–*Bam*HI, 1.1 kb) and 35S (*Eco*RI–*Xho*I; 1.3 kb) from pSLJ2524 (Jones *et al.* 1992), pSU20 (total plasmid, 2.3 kb; Bartolomé *et al.*, 1991), NPTII (*Eco*RI; 1.0 kb) from pCGN1547 (McBride and Summerfelt, 1990) and CHSA (*Sal*I–*Nco*I; 2.4 kb) from VIP119 (Van der Meer *et al.*, 1990).

Polymerase chain reaction (PCR)

PCRs were done as described before (Van der Biezen *et al.*, 1996). The primer sequences were: *act5a* 5′-GCAGATCTTAGGGATGAAAGTAGGATGGG-3′, *act5b* 5′-GCAAGCTTCTCGAGGGGAGAGAGGCAGAGCAGCG-3′, *act3a* 5′-GCAGATCTTAGGGATGAAAACGGTCGG-3′, *act3b* 5′-GCAAGCTTCCGAACAAAAATACCGGTTCCCG-3′, *ac6* 5′-CGCTTGTTCCATGATGACC-3′, *ac7* 5′-GCTGAAGCCTCTTCTAGTCGG-3′, *35s* 5′-ATCTCCACTGACGTAAGGGATGACG-3′ and *spec* 5′-AGTTGAGTCGATACTTCGGCGATCAC-3′ (Isogen Bioscience Amsterdam).

Results

Plants with single copy Ds *containing T-DNA insertions*

The *Ds* element contained the kanamycin (Km) resistance gene *NPTII* for selection of T-DNA or transposon insertions, and was cloned between the *CaMV-35S* promoter and the structural part of the spectinomycin (Sp) resistance gene *TP:SPEC* (Fig. 1). The reporter genes allowed visual monitoring of *Ds* excision and integration in seedlings by simultaneous Sp/Km assays. First, the transposition capacity of the artificial *Ds* element was tested following *Agrobacterium*-mediated transfer of the *Albatross* (*Alb*) T-DNA to a transgenic tobacco line (SLJ10512B) that already contained a *sAc* element. Subsequently, the calluses and shoots were regenerated in the presence of Sp. Tissue that developed green spots and sectors (Sp^R) on the white background (Sp^S) indicated *sAc*-induced excision of *Ds* from the T-DNA (Fig. 2A).

To generate stable *Alb* lines, additional *Agrobacterium* transformations were performed using a non-transgenic

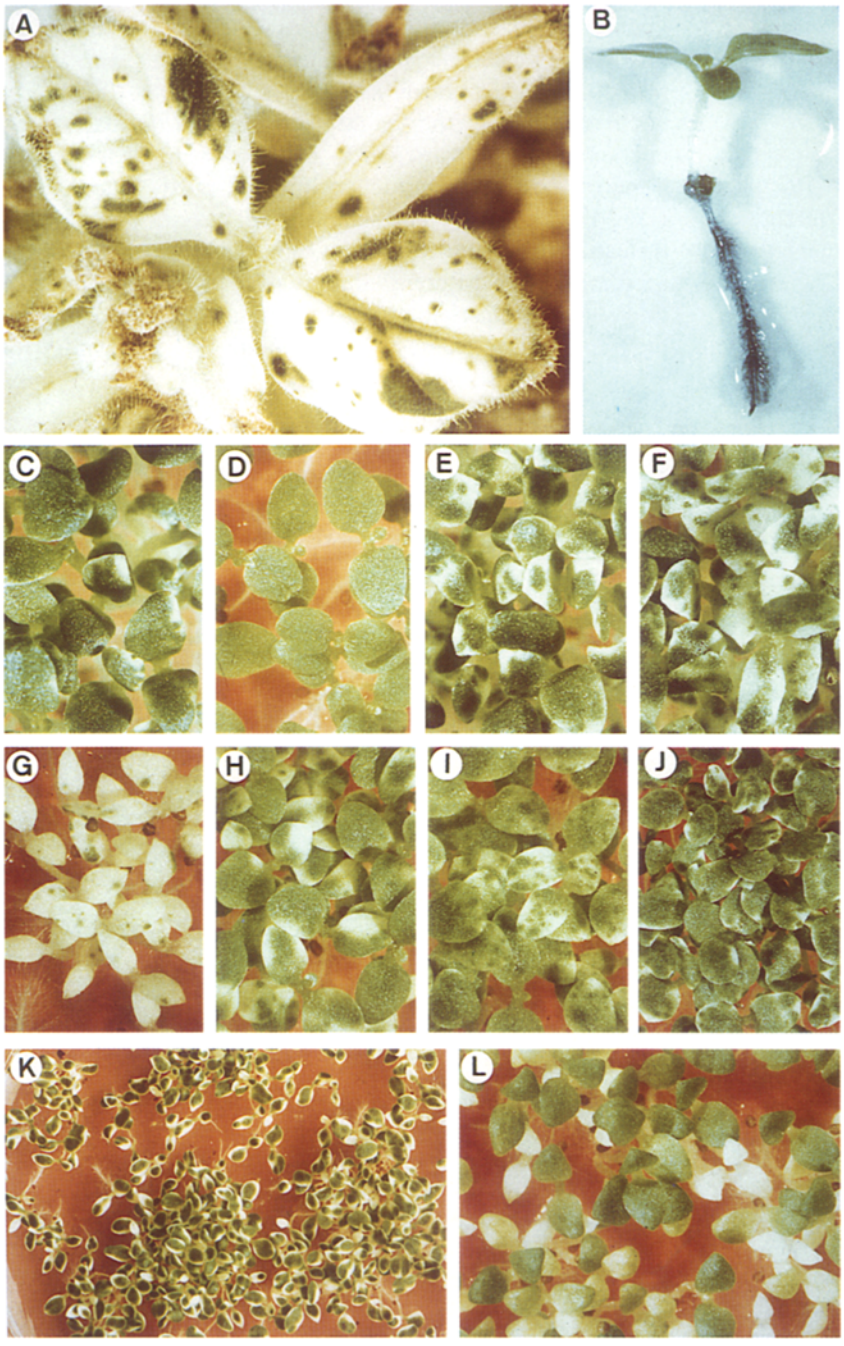

Fig. 2. Visual assays for monitoring *Ds* excision, *Ds* integration and the presence of *sAc*. (A) Regeneration assay for functional analysis of *Ds* transposition. Leafdiscs from a *sAc*-containing plant were transformed with the *Albatross* T-DNA by *Agrobacterium* and calluses and shoots were regenerated in spectinomycin (Sp) medium. Green variegation (Sp^R) indicated excision from the spectinomycin resistance gene (*SPEC*). (B) GUS analysis for the presence of the SLJ10512 constructs carrying *sAc*. The roots of Sp^R/Km^R OC_1 seedlings carrying the T-DNA stained blue after treatment with X-gluc. (C–J) Somatic excision phenotypes of double hemizygous *Ac/Ds* F_1 seedlings derived from the eight *Albatross* lines crossed with plants with the immobile *sAc* construct and germinated on medium with Sp: (C) *Alb*1, (D) *Alb*2, (E) *Alb*3, (F) *Alb*10, (G) *Alb*15, (H) *Alb*17, (I) *Alb*19 and (J) *Alb*20. (K) V1 excision phenotype of double hemizygous F_1 seedlings derived from *Alb*2 and the *chs:Ac* line grown on Sp medium. (L) Distance of *Ds* transposition. The OC_1S_1 seedlings were germinated on Sp/Km medium and segregated for a transposed *Ds* element marked by *NPTII* for Km^R, and the donor site T-DNA marked by *SPEC* for Sp^R. The distance of transposition (cM) was based on the frequency of recombination between *Ds* and the T-DNA. The Sp^R/Km^R seedlings were fully green (FG), all Sp^S seedlings were white (W), and recombination between *Ds* and the T-DNA resulted in Sp^R/Km^S seedlings that were yellow (Y).

tobacco line. In subsequent self progenies of the primary transformants, the number and zygosity of *Alb* T-DNA inserts (*NPTII*) were determined by segregation analysis of Km resistance. Eight independent stable *Alb* transformants with single homozygous T-DNA loci were selected. Southern hybridizations were performed to analyse molecularly the integrity of the T-DNAs. Several enzyme (*Eco*RI, *Hin*d III)/probe (35S, pSU20, NPTII, SPEC) combinations were used (*Hin*dIII/35S analysis shown in Fig. 3, other data not shown). All eight transformants harboured one intact T-DNA copy, whereas *Alb*1 and *Alb*15 contained an additional truncated left part of the T-DNA hybridizing to the 35S probe but not to the other probes. The eight single T-DNA copy *Albatross* transformants allowed subsequent systematic analysis of *Ds* transposition.

Somatic excision of Ds

For *trans*-activation of the *Ds* elements, the *Albatross* lines were crossed with plants that carried different *Ac* transposase constructs: (1) A stabilized *Ac* element (*sAc*, Scofield *et al.*, 1992), (2) an unmodified (wild-type) and transpositionally active *Ac* element (Ac^+, Haring *et al.*, 1989), and (3) a stable *Ac* element in which the transcription of the transposase gene was driven by a heterologous chalcone synthase promoter (*chs*:Ac, Rommens *et al.*, 1992b). Three homozygous siblings of each *Albatross* line were crossed with plants carrying homozygous *Ac* constructs and the F_1 seeds (3–6 capsules per plant) germinated on Sp medium (Fig. 4). The variegation patterns of the seedlings were classified according to the degree of Sp resistance in fully green (FG), highly variegated (HV), medium variegated (MV) and lowly variegated (LV) classes (Fig. 2C–J). Two novel *Ds* variegation patterns were observed that were unique for plants with the *chs*:*Ac* construct. One pattern, designated V1, consisted of 1–3 large green spots of almost the size of the cotyledon (Fig. 2K). The second *chs*:*Ac*-induced variegation pattern (V2) consisted of many (10–20) homogeneously scattered small spots.

For each of the eight different *Albatross* lines, characteristic Sp variegation patterns were observed for a particular *Ac* construct. Although most classes (FG, HV, MV, LV) were represented in all progenies, the lines differed in the proportions of each class. Progeny seedlings from different siblings of a particular *Albatross* line showed remarkable consistency in the composition of the variegation classes. In addition, OC_1 progenies of

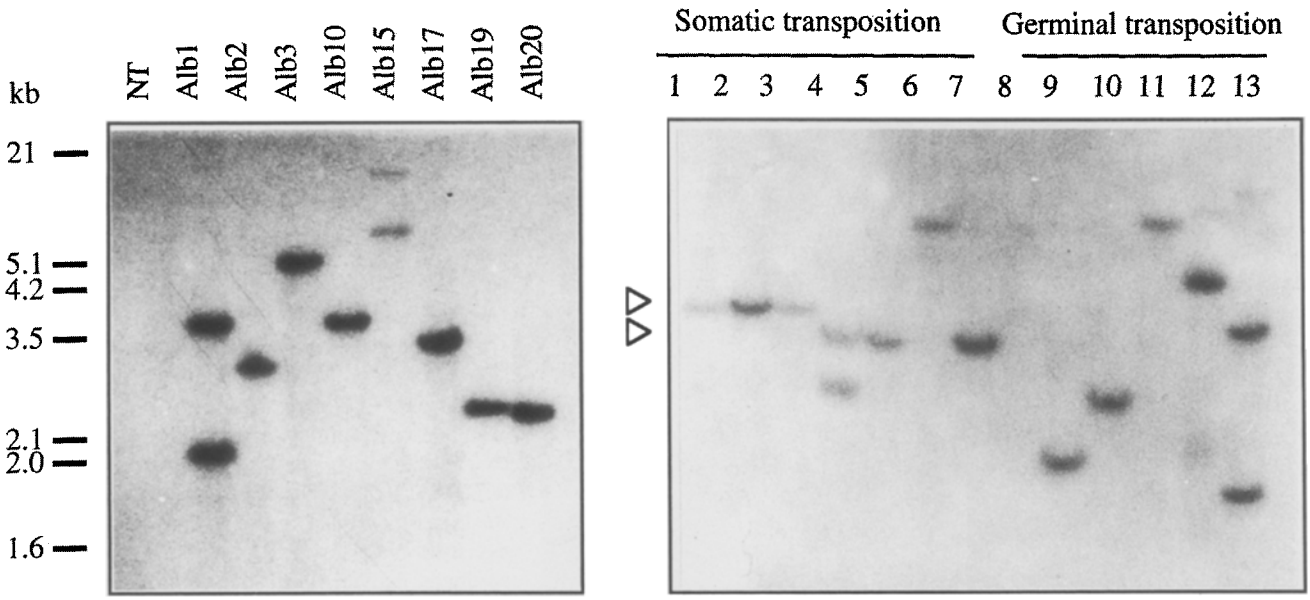

Fig. 3. Southern analyses of parental *Albatross* lines and their F_1 and OC_1 progenies carrying somatic and germinal *Ds* transpositions. Left panel: *Hin*dIII digested DNA of eight primary *Albatross* transformants hybridized with the radiolabelled 35S probe. NT was a non-transgenic plant. Differences in intensity of the bands were due to differences in DNA quantities. Right panel: *Eco*RI digested DNA isolated from *Alb* × *sAc* progenies and hybridized with the pSU20 probe. The seven F_1 plants showing somatic transposition (lanes 1–7) were derived from *Alb*1 (lanes 1–3) and *Alb*2 (lanes 4–7) and selected in Sp assays as variegated (V; lanes 1–5) or fully green (FG; lanes 6 and 7) seedlings. The positions of the *Ds* element at full donor sites (i.e. the T-DNA) for the two *Albatross* lines are indicated by triangles. Bands at other positions indicate transposed *Ds* elements. The five OC_1 plants (lanes 9–13) showing germinal transposition were derived from the *Alb*2 line and selected as FG seedlings in Sp/Km assays and lacked GUS activity ($Sp^R/Km^R/GUS^-$). Bands at different positions indicate unique, germinally transmitted transposed *Ds* elements.

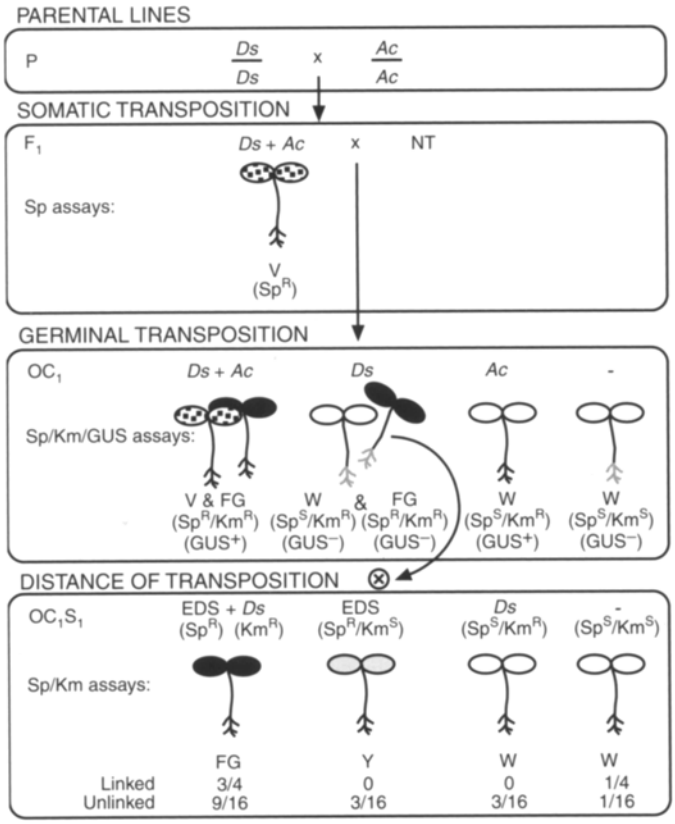

Fig. 4. Crossing scheme used to analyse somatic and germinal *Ds* transposition frequencies and the distance of *Ds* transposition. Eight single-copy homozygous parental *Albatross* lines (P) were selected and crossed to lines that were homozygous for different *Ac* constructs. The progeny seedlings (F_1) were hemizygous for both the *Ds* and the *Ac* elements and, therefore, all could potentially be variegated when germinated on Sp medium. Following outcrossing to a non-transgenic line (NT), the progenies (OC_1) were grown on double selective Sp/Km medium and subjected to GUS analysis. Sp^R/Km^R seedlings were selected that did not contain the *sAc* construct (GUS^-) and, hence, carried a germinally transmitted excised and reinserted *Ds* element. Subsequently, self progenies (OC_1S_1) were analysed for the segregation of the *SPEC* (Sp^R) and *NPTII* (Km^R) genes. The frequency of recombination between *SPEC* and *NPTII* represented the genetic distance between the empty donor site T-DNA (EDS) and *Ds*, respectively, and hence, represented the distance of transposition. FG = fully green, V = variegated, W = white, Y = yellow.

each *Albatross* line showed identical variegation patterns as the F_1, demonstrating that the line specificities of the variegation patterns were heritable (see next section). The low internal variations in the proportion of the variegation classes per *Albatross* line allowed calculation of the mean proportions of each class which represented somatic excision frequencies (Fig. 5).

With *Alb*15 as the exception, the majority of the *Albatross* progenies from *sAc* and Ac^+ crosses consisted of FG and V (any variegated class) seedlings. Only small fractions (0–11%) of the total seedlings were white (W), indicating that *trans*-activation of the *Ds* elements by these *Ac* constructs was generally very effective. Most of the *Albatross* progeny *trans*-activated by the *sAc* element consisted of seedlings of the MV class (81% ± 3%). The *sAc* crosses with *Alb*2 and *Alb*15 were exceptions in that these progenies had a majority of HV (91%) and W seedlings (76%), respectively. Remarkably, the same phenomenon was observed when the Ac^+ element was used for *trans*-activation of *Ds*; however, the degree of variegation was higher. With Ac^+, the majority of progenies consisted of HV seedlings (50% ± 2%), whereas *Alb*2 had mainly FG seedlings (91%) and *Alb*15 progenies mostly MV (45%) and LV seedlings (44%). The *chs*:*Ac* element induced variegation patterns unlike those caused by transposase genes that were transcribed by the original *Ac* promoter. Most F_1 progenies contained both *chs*:*Ac*-induced variegation classes V1 (34% ± 6%) and V2 (33% ± 5%). Also with this construct the progenies of *Alb*2 and *Alb*15 showed extreme high (100% V1) and low (0% V1 and 0% V2) levels of *trans*-activation, respectively. The variegation specificity of the *Albatross* lines was observed in F_1 and OC_1 progenies with all three *Ac* constructs. The *Alb*2 line was shown to contain a *Ds* element that was most sensitive to *trans*-activation by any of the three elements, while the *Alb*15 line carried a *Ds* element that responded lowest to *trans*-activation. As concluded from the degree of variegation, the Ac^+ element was generally more effective in *trans*-activating the *Ds* elements than the *sAc* element. However, excluding the low responding *Alb*15 line, the progenies from the Ac^+ element showed a higher proportion of white (W) seedlings (5% ± 2%) compared to those with the *sAc* element (0.4% ± 0.7%). This might be the result of the loss of the transposase source due to excision of Ac^+ and subsequent failed reinsertion, or due to segregation of Ac^+ after transposition to unlinked sites. Both possibilities did not apply to the immobile *sAc* element.

We used molecular analysis to determine whether excision of *Ds* from the *SPEC* gene corresponded to the phenotypes observed in the seedling assays (FG, V, W). To that end, DNA was isolated from progenies of each type of cross and subjected to PCR and Southern analysis such that empty donor site (EDS) fragments and full donor site (FDS) fragments could potentially be shown. FDS fragments were demonstrated in 32 mature F_1 plants that were V or FG at the seedling stage by PCR (*act5b*/*spec* primers: 0.6 kb) and by Southern hybridization (*Hin*dIII/SPEC probe: 6.5 kb fragments; *Eco*RI/pSU20 probe: ⩾3.3 kb fragments, Fig. 3, right panel). However, the expected EDS fragments (2.6 kb) could only occasionally be demonstrated by Southern hybridization (3 out 32) or by PCR (5 out 32) with the *35s*/*spec* primers (0.5 kb) (data not shown). In contrast, PCR analysis with

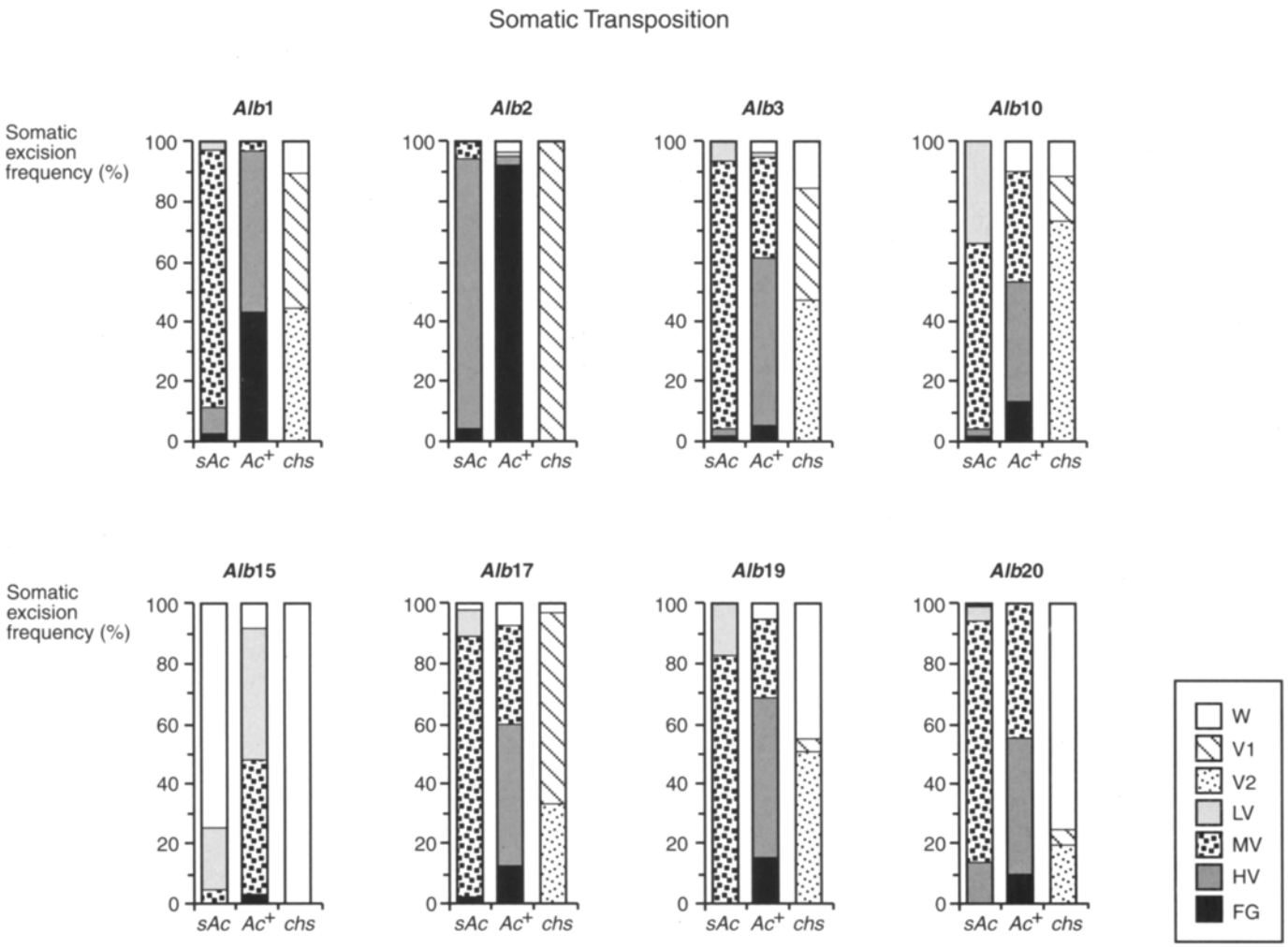

Fig. 5. Frequencies of somatic *Ds* excision from the T-DNAs of eight *Albatross* lines *trans*-activated by *sAc* presented in stack columns. The double hemizygous F_1 seedlings were germinated in the presence of Sp and were classified by the degree of green (Sp^R) variegation. Seedlings with uniform green cotyledons were designated fully green (FG); seedlings with almost entirely green cotyledons but with one or more small white flecks were designated as highly variegated (HV); seedlings with 3–10 distinct green spots were classified as medium variegated (MV); and seedlings that were almost totally white but had 1–2 small green spots were designated as lowly variegated (LV). Seedlings without any visual green variegation were white (W). Seedlings with *chs*:*Ac*-induced variegation patterns were designated V1 when 1–3 large green spots of almost the size of the cotyledon were present, or classified as V2 when the cotyledons were homogeneously scattered by many (10–20) small spots. Each column represents the results of three F_1 plants, of which 3–6 capsules per plant were taken with 100–200 seeds per capsule.

the same primer set on DNA isolated from V and FG seedlings, instead of mature plants, indeed showed in all 24 cases the expected EDS fragments. Apparently, the high degree of somatic *Ds* excision during the formation of the cotyledons in embryogenesis, revealed by the Sp seedling assays, did not coincide with the low degree of somatic *Ds* excision in mature plants. This conclusion is in line with the observation that the degree of Sp resistant variegation at the plant level, revealed by regeneration Sp assays (Fig. 2A), was generally much lower than that observed at the seedling level (Figs 2C–J).

Germinal transposition of Ds

Outcrossing the double hemizygous *Ac*/*Ds* F_1 plants with the non-transgenic line resulted in the OC_1 progenies (Fig. 4). For each *Albatross* line, 3–12 F_1 siblings were used for the outcrosses and 3–13 pollinations were made per plant. The OC_1 seedlings were germinated on double selective medium, containing both Sp and Km. These Sp/Km assays showed similar proportions of variegation classes as observed in the F_1, indicating that the variegation specificity of the *Albatross* lines was heritable (data not shown). Also in the OC_1, the *trans*-activation of *Ds* was generally very effective because in most *Albatross*

progenies the expected 1/4 of the seedlings (carrying both *Ac* and *Ds*) was variegated. As in the F_1, *Alb*15 crossed with the *sAc* line was the only exception in that only a small fraction of the OC_1 seedlings was LV (<5%) and the others were W.

Transposition events in F_1 cell lineages that had been transmitted through the germline to the OC_1 resulted in FG seedlings when germinated on Sp/Km medium. The frequency of germinal transposition could only unmistakeably be calculated when FG seedlings (Sp^R/Km^R) were selected that lacked the *Ac* transposase source and, therefore, could not be the consequence of somatic excision, nor could Km resistance be conferred by the *Ac* T-DNA. This procedure was followed only for *Ds* elements *trans*-activated by the *sAc* element (Fig. 6). For molecular analysis, DNA was isolated from mature OC_1 plants that were FG (Sp^R/Km^R) at the seedling stage and that lacked the *sAc* element (GUS^-). PCR with *35s/spec* primers showed the EDS fragments and, as expected, the FDS fragments (*act5b/spec*) and the *Ac*-specific fragments (*ac6/ac7*) could not be demonstrated. Progeny testing by Sp/Km seedling assays showed that 136 of the 145 OC_1 plants segregated for Sp and Km resistance in the OC_1S_1 (see next section). Southern analysis showed that nine OC_1 plants did not contain a *Ds* element (*NPTII*) and apparently escaped the selection. The majority (78%) of the FG seedlings (Sp^R/Km^R) were GUS^+ (Table 1). It would be expected that seedlings with germinal *Ds* transpositions segregated independently (1:1) from the *sAc* T-DNA (*GUS*). Therefore, the surplus of FG/GUS^+ seedlings was most likely the result of somatic excision. Similar proportions (on average, 1%) of somatically-induced FG seedlings had been observed in the F_1 (Fig. 5). The *Alb*2 and *Alb*15 progenies showed the highest (0.95%) and the lowest (0.15%) germinal transposition frequencies, respectively (Table 1). The same *Albatross* lines showed similar results in the somatic transposition frequencies. The possibility that the low average germinal *Ds* transposition frequency (0.37%) of all *Albatross* lines *trans*-activated by the *sAc* element was caused by a high incidence of failed *Ds* reinsertions was tested by seedling assays with only Sp in

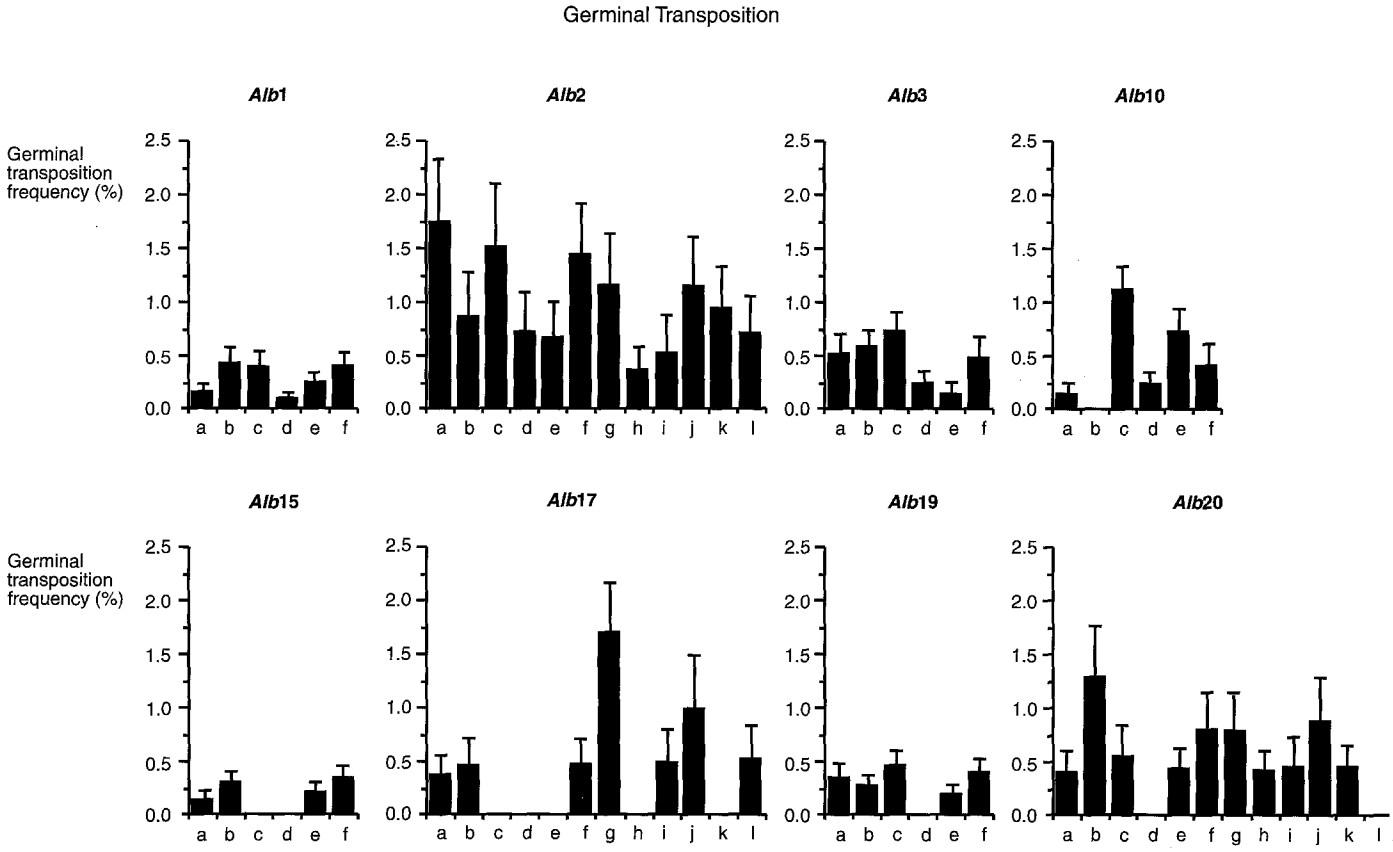

Fig. 6. Frequency of germinal *Ds* transpositions from the T-DNAs of eight *Albatross* lines *trans*-activated by *sAc* as determined in outcrossed progenies (OC_1). Sp^R/Km^R seedlings were selected that did not contain the *sAc* T-DNA (GUS^-) and, hence, unmistakably carried a germinally transmitted excised and reinserted *Ds* element. The results are shown for 6–12 siblings per *Albatross* line (13 000–28 000 seeds).

Table 1. Summary of the germinal *Ds* transposition frequencies in eight *Albatross* lines *trans*-activated by the immobile *sAc* element

Ds *line*	*Number of F_1 plants*	*Number of OC_1 capsules*	*FG seedlings*[a]		*Variegated seedlings*[b]	*Germinal transposition frequency (% ± sd)*
			GUS$^-$	GUS$^+$		
*Alb*1	6	70	18	80	6,906	0.26 ± 0.06
*Alb*2	12	34	30	100	3,173	0.95 ± 0.17
*Alb*3	6	49	21	42	4,782	0.44 ± 0.09
*Alb*10	6	44	18	53	5,169	0.35 ± 0.08
*Alb*15	6	47	7	18	4,602[b]	0.15 ± 0.05
*Alb*17	12	33	13	51	3,211	0.41 ± 0.11
*Alb*19	6	49	14	80	6,026	0.23 ± 0.06
*Alb*20	12	36	15	65	3,308	0.45 ± 0.12
Overall	66	362	136	489	37,177	0.37 ± 0.03

[a]For unambiguous identification of OC_1 plants that carried germinally transmitted transposed *Ds* elements, fully green (FG) seedlings (Sp^R/Km^R) were identified that did not contain the *sAc* T-DNA (GUS^-); [b]the number of seedlings that potentially could carry a germinally transmitted transposed *Ds* element (1/2 of the OC_1) was based on the number of variegated seedlings (1/4 of the OC_1); the population sizes of *Alb*15 progenies were determined by counting all seedlings.

the medium. However, the numbers of FG seedlings were not significantly higher than with Km present in the medium, indicating that *Ds* excision was generally coupled to reinsertion. Additionally, the reciprocal outcrosses showed no differences in the frequency of transmission of transposed *Ds* elements through the female or male gametes (data not shown). The Ac^+ line generally produced much higher frequencies of FG seedlings (1%–100%) than most of the *sAc* progenies (0.3%–2.0%). Similarly as in the F_1, the OC_1 progenies of *Alb*15 × Ac^+ showed a low frequency of FG seedlings (1.4%). In progenies of the *chs*:*Ac* line only seedlings with the V1 and V2 phenotypes were observed. Out of an estimated number of 10,000 seedlings (3 F_1 siblings for each 8 *Albatross* lines, 3 outcrosses per plant), no FG seedlings were observed. Therefore, it is concluded that *trans*-activation of the *Albatross* lines by the *chs*:*Ac* element did not result in germinal transposition.

Germinal transposition frequencies do not necessarily reflect the proportion of unique *Ds* transposition events. Clonal propagation of transposition events in somatic F_1 tissues prior to the formation of gametes could lead to transmission of that single event to several OC_1 seedlings. To determine the incidence of independent unique *Ds* transpositions, the reproductive cell lineages were followed by recording the position of each pollinated F_1 flower. The occurrence of independent germinal *Ds* transposition was established by investigating whether *Ds* elements were inserted into different DNA restriction fragments. To analyse these new insertion site (NIS) fragments, DNA was digested (*Eco* RI or *Hind* III) and analysed by Southern hybridization with *Ds*-specific probes (pSU20 or NPTII) (Fig. 3, right panel). NIS fragments of different sizes indicated that the *Ds* elements were reinserted at different positions in the genome. Out of the 136 OC_1 plants with germinally transmitted transposed *Ds* elements, 18 were obtained that were derived from F_1 plants that generated only one such OC_1 plant and, by definition, carried unique transposition events. Comparison of NIS fragments between siblings showed that similar NIS fragments were only observed for OC_1 plants that were derived from the same seed capsule, i.e. F_1 flower. Twenty OC_1 plants (20/118 = 17%) had similar NIS fragments as one or two siblings and, therefore, in principle carried the same premeiotic transposition event. Thus, in total, 116 OC_1 plants were identified that carried unique *Ds* transposition events. Three of these contained two NIS fragments and, hence, contained two transposed *Ds* elements.

Distance of Ds *transposition*

A total of 113 OC_1 plants was identified that carried single, unique, transposed and germinally transmitted *Ds* elements. To determine the distance of transposition of these *Ds* elements, OC_1S_1 progenies (200–400 seedlings) were analysed for segregation of Km and Sp resistance that were conferred by the *Ds* element (*NPTII*) and the T-DNA (*SPEC*), respectively (Fig. 4). If significant linkage was observed ($p < 0.01$), recombinant fractions were calculated and converted to genetic distance in cM. Each *Albatross* line showed differences in the distribution of transposed *Ds* elements (Fig. 7). The transposition patterns mainly varied in the dispersal of loosely linked transpositions. Notwithstanding the variation between *Ds* lines, generally each pattern showed similar characteristics (Table 2). In most *Albatross* lines the majority of *Ds* elements transposed closely linked (0 cM) and unlinked

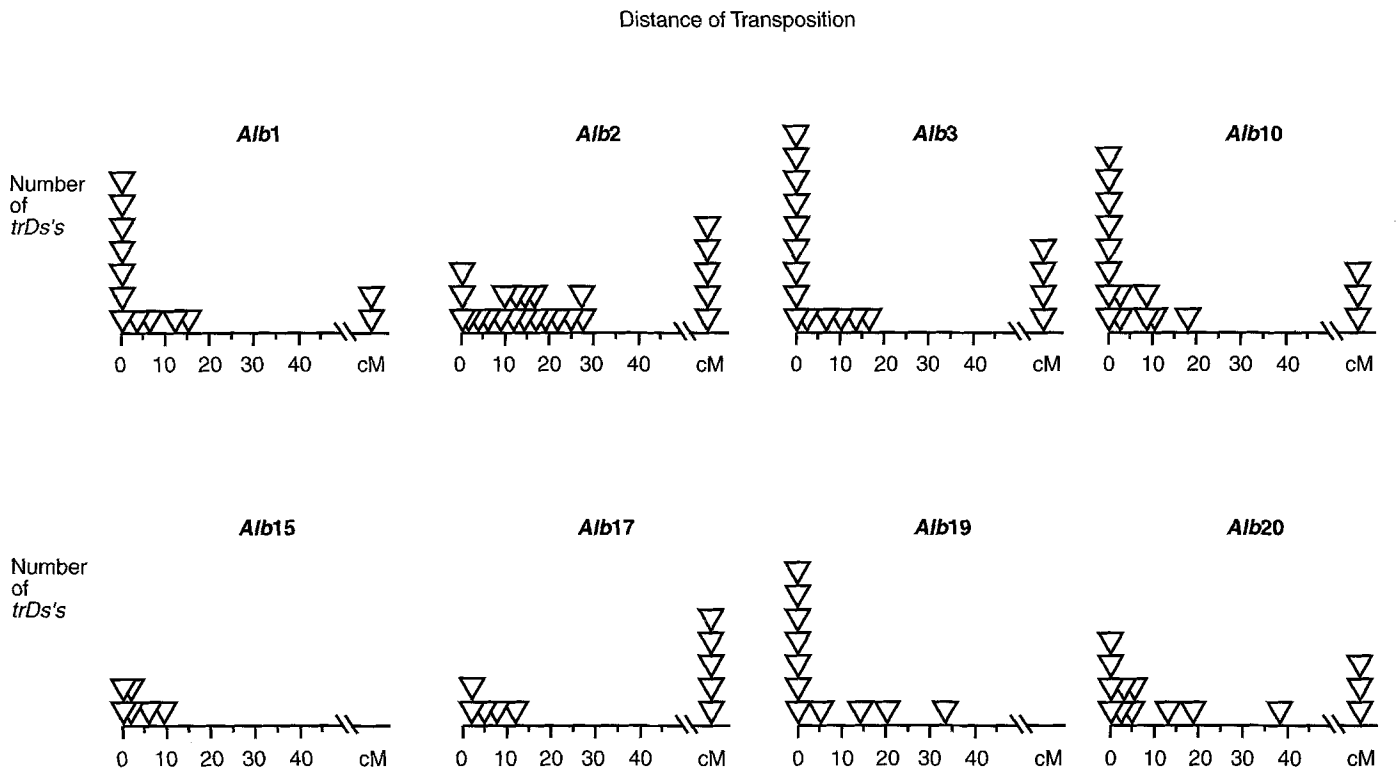

Fig. 7. Distance of transposition from the T-DNAs of eight *Albatross* lines *trans*-activated by *sAc* as determined in OC_1S_1 progenies. Distances were expressed in cM using Haldane's mapping function to convert the recombination frequencies between the *Ds* elements and the original donor site T-DNAs ($P < 0.01$). The *Ds* element was marked by the *NPTII* gene and the T-DNA was marked by the *SPEC* gene. The segregation of *Ds* and the T-DNA was analysed in Sp/Km assays, where Sp^R/Km^R seedlings were fully green (FG), Sp^S were white (W), and Sp^R/Km^S seedlings, carrying a recombination event, were yellow (Y).

Table 2. Summary of the distance of *Ds* transpositions in eight *Albatross* lines *trans*-activated by the immobile *sAc* element

		Linked transpositions[a]				*Unlinked transpositions*	
Ds line	*Number of unique transposed* Dss	*Number*	*%*	*Mean distance (cM±sd)*	*Range (cM)*	*Number*	*%*
*Alb*1	13	11	85	3.4 ± 2.4	0.0–15.3	2	15
*Alb*2	24	19	79	12.6 ± 3.0	0.0–26.9	5	21
*Alb*3	18	14	78	3.6 ± 2.4	0.0–15.8	4	22
*Alb*10	17	14	82	3.5 ± 2.4	0.0–17.4	3	18
*Alb*15	6	6	100	2.9 ± 2.0	0.0–9.3	0	0
*Alb*17	10	5	50	5.8 ± 2.2	1.6–4.9	5	50
*Alb*19	11	11	100	6.6 ± 3.3	0.0–33.0	0	0
*Alb*20	14	11	79	7.4 ± 3.4	0.0–37.7	3	21
Overall	113	91	81	6.4 ± 2.9	0.0–37.7	22	19

[a]If significant linkage was observed ($p = <0.01$ if $\chi^2 > 11.32$) recombinant fractions were converted to cM by Haldane's mapping function.

(>40 cM) to the original T-DNA. The exceptional line was *Alb2* of which the majority of transposed *Ds* elements were mapped at positions between 2–27 cM. The average frequencies of linked (<40 cM, 81%) and unlinked (19%) transpositions were comparable in most *Albatross* lines. The mean transposition distance of all *Albatross* lines was 6.4 cM; of which the *Alb*2 line significantly deviated with 12.7 cM. The overall frequency distribution of all 113 transposition distances showed that 35% could not be separated from the donor site, and that the number of transposed *Ds* elements gradually decreased with increasing distances of transposition (Fig. 8).

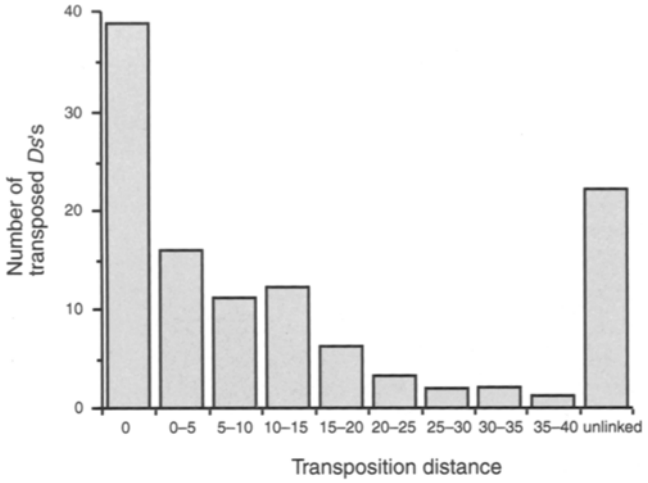

Fig. 8. Frequency distribution of the total *Ds* transposition distances of all eight *Albatross* lines *trans*-activated by *sAc*.

Discussion

Somatic excision of Ds

We systematically characterized the behaviour of an artificial *Ds* element in eight tobacco lines *trans*-activated by three different *Ac* elements. Since the *Ds* elements remained immobile until fertilization, the timing and frequency of somatic excisions during embryogenesis could efficiently be monitored visually in seedling assays. The high proportions of seedlings with excised *Ds* elements showed that *trans*-activation during cotyledon development was generally very effective. Furthermore, each *Ds* line showed characteristic spectinomycin resistant (Sp^R) variegation patterns that were inherited to following generations. The lines differed in the proportion of seedlings with Sp^R green spots of different size and number, which were a consequence of the timing and frequency of *Ds* excisions during development of the cotyledons, respectively. Variability in *Ds* excision patterns has also been observed in *Arabidopsis* and lettuce (Bancroft and Dean, 1993a; Yang *et al.*, 1993). The unmodified (wild-type) and transpositionally active Ac^+ element proved to be more effective in *trans*-activating the *Ds* elements than the immobile *sAc* element. This positive effect on the *Ds* transposition frequency could be the result of an increased number of Ac^+ copies, or could be due to a more *trans*-active Ac^+ element (Jones *et al.*, 1991). The lower *trans*-activation effectiveness of the *sAc* element was probably not the result of the sequence modification because the 177 bp terminal deletion does not include the transposase gene (Keller *et al.*, 1993).

The *chs*:*Ac* element induced different *Ds* variegation patterns (V1, V2) than the *Ac* elements that were under the control of the endogenous promoter. The V1 class consisted of seedlings with 1–3 large green spots as a consequence of *Ds* excision in a few cells early in cotyledon development. These spots arose from a few progenitor Sp^R cells in the center of the cotyledon and did not include the edges. The V2 class, on the other hand, consisted of a large number of small green spots resulting from *Ds* excisions in many cells late in cotyledon development. The occurrence of two types of *chsA* promoter-induced variegation patterns suggested that this promoter was active twice during cotyledon development. However, seedlings were only identified that showed either the V1 or the V2 pattern, indicating that during development of an individual seedling the *chsA* promoter was active only once. Alternatively, the V1 pattern could be the consequence of a few early *Ds* excisions until high transposase levels became inhibitory (Scofield *et al.*, 1993). The activity of the *chsA* promoter in *P. hybrida* seedlings has been demonstrated by *GUS* fusions (Koes *et al.*, 1990; Van der Meer *et al.*, 1990). Using other *promoter*:*Ac* transposase gene fusions (*nos*:*Ac*, *ocs*:*Ac*, *CaMV-35S*:*Ac*) in experiments with similar visual *Ds* excision assays (streptomycin resistance, *SPT*) distinct variegation patterns have also been observed, unlike those caused by the immobile *sAc* or Ac^+ elements (Scofield *et al.*, 1992).

Germinal transposition of Ds

In contrast to developing seedlings, *Ds* excisions took place infrequently in mature plants. Most likely, the low somatic *Ds* activity resulted in accompanying low frequencies by which transposed *Ds*s were germinally transmitted (0.37%). This suggestion is supported by the observation that the same *Ds* lines showed the highest (*Alb*2) and lowest (*Alb*15) values in both somatic and germinal *Ds* transposition frequencies. Previously, it has been shown that *trans*-activation of another *Ds* line by the same and other *sAc* lines resulted in similar low germinal transposition frequencies (0.38%, Scofield *et al.*, 1992). The high incidence of independent transposition events (83%) indicates a late timing of transposition, i.e. during development of the generative meristem, prior to gametogenesis. Following unlinked transposition of *Ds*, only half of the progeny seedlings (Sp^R/Km^R) carrying that event could be visually identified by the fully green (FG) appearance, and the others (Sp^S/Km^R) were white (W). Therefore, the phenotypic identification of seedlings carrying transposed *Ds* elements included a selection bias for genetically linked transpositions. On the basis of the proportions of unlinked and loosely linked *Ds* transposition in 113 of the 136 selected seedlings (Table 2), it was estimated that a maximum of 30 seedlings with unlinked transpositions could have escaped the selection. Consequently, incorporating these non-selected seedlings would increase the overall germinal transposition frequency to 0.45% ([30 + 136]/37177; Table 1).

The *trans*-activation of *Ds* by the *chs*:*Ac* element did not result in germinally transmitted transposition events. Most likely the *chsA* promoter did not express the *Ac* transposase gene in germinal cell lineages (L2) because it is predominantly active in L1 tissues (Koes *et al.*, 1990; Van der Meer *et al.*, 1990). No differences were observed in progenies from the reciprocal outcrosses, excluding differences in the activity of the *chsA* promoter between male and female organs. In other *promoter*:*Ac* fusion experiments in tobacco, the heterologous promoters induced distinct germinal *Ds* excision frequencies: 7% for the *CaMV-35S*:*Ac* fusion, 0.87% for the *ocs*:*Ac* fusion and, 0.09% for the *nos*:*Ac* fusion (Scofield *et al.*, 1992). Remarkably, in contrast to these low germinal transposition frequencies of *Ds* elements (0.0–7%), much higher germinal excision frequencies (4–80%) were reported for wild-type Ac^+ elements in tobacco. However, comparison of the transposition rates is complicated by the different *Ac* dosages, and different transposition assays applied in these studies (Hehl and Baker, 1990; Jones *et al.*, 1991; Fitzmaurice *et al.*, 1992; Keller *et al.*, 1993). In most other heterologous hosts, the activities of Ac^+ and *Ds* cannot be compared directly because the regulatory sequences of the *Ac* component were often modified when used in combination with *Ds* (Bancroft *et al.*, 1992; Ellis *et al.*, 1992; Yang *et al.*, 1993).

Distance of Ds *transposition*

Although the 113 *Ds* transpositions from the eight T-DNA loci in tobacco showed a general pattern, their distributions varied in the dispersal of loosely linked transpositions. This variability in transposition pattern has been observed before for *Ac* in tobacco and for *Ds* in *Arabidopsis* (Dooner *et al.*, 1991; Bancroft and Dean, 1993b). Following simultaneous selection for seedlings with transposed *Ds* elements, half of the seedlings with unlinked transpositions were not identified, and as a consequence, the proportions of unlinked transpositions were possibly underestimated. However, the *Ds* elements in all tobacco lines showed a strong tendency for transposition to genetically closely linked sites. On average, 81% of the *Ds* elements transposed within 40 cM, and 58% transposed within a 10 cM distance. Similar observations have been made for *Ac* in tobacco: 72% within 40 cM, 45% within 10 cM (Jones *et al.*, 1990; Dooner *et al.*, 1991). In addition, the frequencies of linked (<40 cM) *Ds* transposition distances in *Arabidopsis* and tomato, also determined by segregation analysis of the T-DNA and the *Ds*, were comparable: 68% and 84%, respectively (Bancroft and Dean, 1993b; Carroll *et al.*, 1995).

Implications of this study

For the further development of transposon tagging strategies in heterologous hosts using the *Ac*/*Ds* system, the parameters involved should be optimized. To increase the transposon-induced mutation frequency, high germinal *Ds* transposition frequencies should be established by modification of the *Ac* transposase sources. Higher transposase levels produced by using heterologous promoters lead to increased transposition frequencies up to a certain threshold when transposition is inhibited (Scofield *et al.*, 1993). With respect to these dosage effects, transposase expression should be regulated within a narrow range. Simultaneously, high frequencies of independent transpositions should be achieved by inducing transposition in late germline cells. Therefore, *Ds* elements should be *trans*-activated in the generative meristem just prior to the formation of gametes by regulated expression of the *Ac* transposase using tissue-specific or inducible promoters (Balcells *et al.*, 1994; Charng *et al.*, 1995). The *Ds* lines with the visual transposition reporters that were generated and characterised in this study will be useful for testing novel *Ac* constructs.

In transposon tagging experiments with particular *Ds* lines, the variability in the frequency and distances of germinal *Ds* transpositions can result in unexpected lower mutation frequencies. Variation in the expression of transgenes is presumed to be attributed to position effects due to differences in the integration site of the T-DNA. Supposedly, the chromatin conformation at the site of integration influences the binding of transcription factors and other factors. Indeed, the presence of chromatin matrix-associated regions (MARs) around the transgenes has been shown to reduce the variability of their expression in tobacco (Mlnàrovà *et al.*, 1994). It is suggested that the variability in *Ds* transposition behaviour is caused by differences in the timing and frequency of binding of the *Ac* transposase and other factors due to the effects of the genomic position. This hypothesis may be tested by cloning MARs around the *Ds* elements. Likewise, the establishment of artificial chromatin domains around the T-DNA loci may result in the *Ds* elements being less susceptible to influences of chromatin in the topological vicinity of the integration site.

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